

# Invasion of vascular cells *in vitro* by *Porphyromonas endodontalis*

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## Abstract

**Dorn BR, Harris LJ, Wujick CT, Vertucci FJ, Progulsk-Fox A.** Invasion of vascular cells *in vitro* by *Porphyromonas endodontalis*. *International Endodontic Journal*, **35**, 366–371, 2002.

**Aim** The objective of this study was to determine whether laboratory strains and clinical isolates of microorganisms associated with root canal infections can invade primary cultures of cardiovascular cells.

**Methodology** Quantitative levels of bacterial invasion of human coronary artery endothelial cells (HCAEC) and coronary artery smooth muscle cells (CASMC) were measured using a standard antibiotic protection assay. Transmission electron microscopy was

used to confirm and visualize internalization within the vascular cells.

**Results** Of the laboratory and clinical strains tested, only *P. endodontalis* ATCC 35406 was invasive in an antibiotic protection assay using HCAEC and CASMC. Invasion of *P. endodontalis* ATCC 35406 was confirmed by transmission electron microscopy.

**Discussion** Certain microorganisms associated with endodontic infections are invasive. If bacterial invasion of the vasculature contributes to the pathogenesis of cardiovascular disease, then microorganisms in the pulp chamber represent potential pathogens.

**Keywords:** coronary cells, endodontic, invasion, *Porphyromonas endodontalis*, *Prevotella*.

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## Introduction

There is an emerging paradigm shift from coronary heart disease (CHD) having a purely hereditary/nutritional causation to having an infectious component (Libby *et al.* 1997, Coles *et al.* 1998, Nieto 1998). Some potential pathogens include *Chlamydia pneumoniae*, *Helicobacter pylori*, Cytomegalovirus, and oral bacteria (Mattila *et al.* 1998, Epstein *et al.* 1999). Some epidemiological studies have demonstrated an association between periodontal disease and coronary heart disease (DeStefano *et al.* 1993, Mattila *et al.* 1993, Mattila *et al.* 1995, Beck *et al.* 1996, Arbes *et al.* 1999, Morrison *et al.* 1999). Conversely, several studies have reported no significant associations between oral disease and CHD (Joshupura *et al.* 1996, Hujoel *et al.* 2000, Mattila *et al.* 2000). Two of the

latter studies that reported no significant association did find associations between the two diseases in younger age groups. Thus, there may be no overall association, but there may be associations within subsets of the population, for example, younger age groups and specific human genotypes (Beck *et al.* 2000). Invasion of the cells of the arterial wall by oral bacteria could represent the injury that either initiates and/or more probably exacerbates atherogenesis.

Transient bacteraemias occur in patients after tooth-brushing, mastication, extraction of teeth, periodontal therapy, and endodontic treatment (Sconyers *et al.* 1973, Silver *et al.* 1977, Carroll & Sebor 1980, Debelian *et al.* 1995), and this dissemination of oral bacteria may be a possible route of infection to those arterial tissues. Oral bacteria have been found in atherosclerotic plaques (Chiu 1999, Haraszthy *et al.* 2000). A few periodontal pathogens have been reported to invade oral epithelial tissues. The periodontal pathogens *Porphyromonas gingivalis* and *Prevotella intermedia* also invade primary

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cultures of human coronary artery endothelial cells (HCAEC) and coronary artery smooth muscle cells (CASMC) (Dorn *et al.* 1999).

Anaerobic bacteria are found within root canal infections and may have a role in the pathogenesis of pulpal disease (Assed *et al.* 1996). Endodontic infections are frequently associated with black-pigmented bacteria (BPB) as a group. *Porphyromonas* and *Prevotella* spp., both BPBs, occur within 79% of endodontic lesions (Haapasalo 1989), and *P. nigrescens* is the most common of the BPBs in the pulp canal (van Winkelhoff *et al.* 1988, Bae *et al.* 1997). Several periodontal pathogens, including *P. gingivalis* and *P. intermedia*, are frequently found in root canal lesions. Additionally, some microorganisms associated with endodontic lesions (*Porphyromonas endodontalis* and *Prevotella nigrescens*) are closely related to invasive periodontal pathogens. Therefore, the objective of this study was to determine whether microorganisms associated with endodontic lesions have the ability to invade primary cultures of human coronary artery endothelial cells and coronary artery smooth muscle cells. In addition to the laboratory strains, isolated bacterial strains from root canal lesions from patients at the University of Florida, USA were tested for invasive ability. The bacteria from root canal infections have the potential to be involved in the development of atherosclerosis and CHD since they have access to the systemic circulation.

## Materials and methods

### Bacterial and cell culture

In addition to laboratory strains, black-pigmented bacteria (BPB) were isolated from patients undergoing root canal treatment as described previously (Debelian *et al.* 1995). Strains of BPBs (Table 1) were grown on tryptic soy agar (Difco Laboratories, Detroit, MI, USA) supplemented with 5.0% sheep blood (Lampire Biological Laboratories, Pipersville, PA, USA), 0.5% yeast extract (Difco), haemin ( $5 \mu\text{g mL}^{-1}$ ), and vitamin K ( $5 \mu\text{g mL}^{-1}$ ). Overnight cultures of BPB were grown in brain heart infusion (BHI) broth (Difco) supplemented with 0.5% yeast extract, 0.1% cysteine (Sigma Chemical Co., St. Louis, MO, USA), haemin ( $5 \mu\text{g mL}^{-1}$ ), and vitamin K ( $5 \mu\text{g mL}^{-1}$ ) under anaerobic conditions. *E. coli* MC1061 (a gift of A. S. Bleiweis) was grown in Luria-Bertani (LB) medium consisting of Bacto Tryptone ( $10 \text{ g L}^{-1}$ ; Difco), Bacto yeast extract ( $5 \text{ g L}^{-1}$ ), and NaCl ( $10 \text{ g L}^{-1}$ ).

The HCAEC were maintained in Microvascular Endothelial Growth Medium-2 (EGM-2; Clonetics, Inc.,

**Table 1** Bacterial strains used

| Bacterial strains                 | Source                               |
|-----------------------------------|--------------------------------------|
| <i>E. coli</i> MC1061             | A. S. Bleiweis <sup>a</sup>          |
| <i>P. endodontalis</i> ATCC 35406 | D. Grenier <sup>b</sup>              |
| <i>P. endodontalis</i> H11a-e     | D. Grenier                           |
| <i>P. endodontalis</i> R-41       | D. Grenier                           |
| <i>P. gingivalis</i> 381          | SUNY-Buffalo Collection <sup>c</sup> |
| <i>P. intermedia</i> 27           | PDRC Collection <sup>d</sup>         |
| <i>P. intermedia</i> FAB8         | This study                           |
| <i>P. loescheii</i> FAB1          | This study                           |
| <i>P. nigrescens</i> ATCC 9336    | PDRC Collection                      |
| <i>P. tanneriae</i> FAB1          | This study                           |
| <i>P. tanneriae</i> FAB8          | This study                           |

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San Diego, CA, USA) consisting of endothelial cell basal medium-2 supplemented with fetal bovine serum, hydrocortisone, human recombinant fibroblast growth factor, vascular endothelial growth factor, recombinant insulin growth factor-1, ascorbic acid, human recombinant epidermal growth factor, gentamicin, and amphotericin (Clonetics). The CASMC (Clonetics) were maintained in smooth muscle growth medium (SmGM-2) consisting of smooth muscle basal medium-2 supplemented with insulin, human recombinant fibroblast growth factor, fetal bovine serum, human recombinant epidermal growth factor, gentamicin, and amphotericin (Clonetics). Cells were cultured in 75-cm<sup>2</sup> flasks at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. Both the HCAEC and CASMC were obtained cryopreserved third passage and were passaged an additional two or three times before use.

### Sampling from the root canal

The protocol for collection of bacteria from infected root canals was that described by Debelian *et al.* (1995). Clinical isolates from infected root canals were identified as described by Moore *et al.* (1994) using the MIDI identification system (Microbial ID, Inc., Newark, DE, USA).

### Invasion assay

For the invasion assays, the bacteria were grown in BHI broth, centrifuged at  $3500 \times g$ , and resuspended in antibiotic-free media to a concentration of  $10^7$  cells mL<sup>-1</sup> as determined spectrophotometrically (Shimadzu UV-1201, VWR, Marietta, GA, USA). Approximately  $10^5$  human cells per well in a 24-well tissue culture plate

were washed three times with phosphate-buffered saline (PBS) prior to incubation with 1.0 mL of the bacterial suspension at 37 °C aerobically for 90 min. In order to more closely approximate *in vivo* conditions, the bacteria were not centrifuged onto the cells to promote intimate contact. The media were aspirated off infected cells after 90 min, and the cells were washed three times with PBS. Medium containing gentamicin (300 µg mL<sup>-1</sup>) and metronidazole (200 µg mL<sup>-1</sup>) was then added to each well to kill any extracellular bacteria, and the plates were incubated for 60 min aerobically at 37 °C. Finally, the media were removed, the cells washed three times with PBS, and lysed by a 20-min incubation with 1.0 mL sterile distilled water at 37 °C under aerobic conditions. Dilutions of the lysates of cells infected with BPBs were plated in triplicate on Tryptic Soy Agar (Difco) plates supplemented with 5.0% sheep blood, 0.5% yeast extract, haemin (5 µg mL<sup>-1</sup>), and vitamin K (5 µg mL<sup>-1</sup>) and cultured anaerobically. The dilutions of the lysates of *E. coli* MC1061 were cultured on LB plates at 37 °C aerobically. Colony forming units (CFU) of invasive bacteria were then enumerated. Each assay was performed in duplicate wells and independently at least three times. Viability of vascular cells was examined by trypan blue exclusion.

Control experiments demonstrated that extracellular bacteria were killed at these antibiotic concentrations. Invasive bacteria not protected within human cells had CFUs lower than the non-invasive strains. The CFU of *P. gingivalis* 381 decreased by four orders of magnitude.

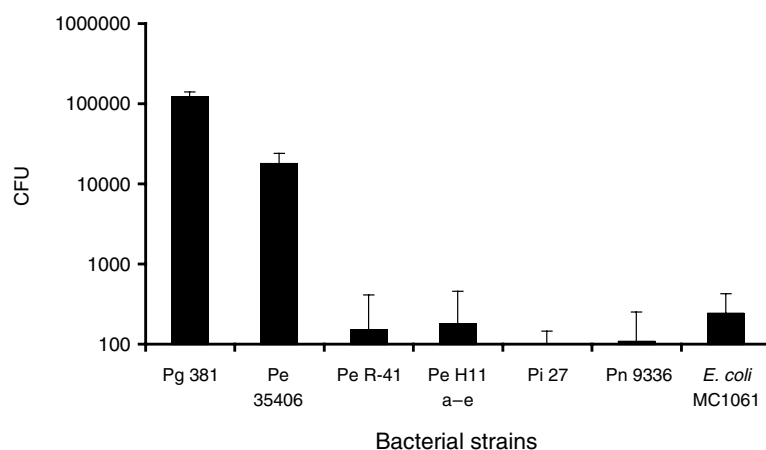
#### Transmission electron microscopy

Following the 90 min incubation of eukaryotic cells with bacteria, the cells were washed two times with PBS, fixed

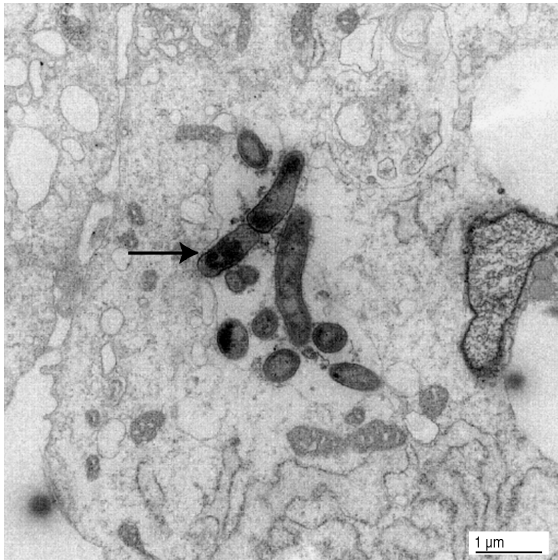
in 2% PBS buffered glutaraldehyde at room temperature for 1 h, centrifuged for 5 min at 200 × *g*, and washed with PBS (pH 7.3). Three drops of 3% low-gelling agarose were then added to the pellet and allowed to solidify at 4 °C for 10 min. The agarose-embedded pellet was then washed twice for 10 min in PBS, incubated in 1% osmium tetroxide for 1 h, and washed three times in distilled H<sub>2</sub>O. The washed cell pellets were dehydrated in a graded series of ethanol and stained overnight *en bloc* with 2% uranyl acetate. Finally, the pellets were infiltrated and embedded in EM Bed-812 (Electron Microscopy Sciences, Ft. Washington, PA, USA). Thin sections were cut, poststained with uranyl acetate and lead citrate, and examined in a Hitachi 7000 transmission electron microscope.

#### Results

Five laboratory strains of microorganisms (*P. endodontalis* strain 35406, *P. endodontalis* strain R-41, *P. endodontalis* strain H11 a–e, *P. intermedia* strain 27, and *P. nigrescens* strain 9336) associated with endodontic infections were tested for the ability to invade primary cultures of endothelial and smooth muscle cells. Of these, only *P. endodontalis* ATCC 35406 was invasive (the CFU were higher than non-invasive *E. coli* MC1061) in the antibiotic protection assay (Fig. 1). The CFU of strain 35406 recovered following the antibiotic incubation was  $1.8 \pm 0.6 \times 10^4$  in HCAEC. Although the invasive ability of *P. endodontalis* ATCC 35406 was approximately 10-fold less than *P. gingivalis* 381, it is considered moderately invasive according to the classification previously described (Dorn *et al.* 2000). Invasion was confirmed by transmission electron microscopy (Fig. 2). Although *P. endodontalis* is closely related to *P. gingivalis*, its intracellular location following invasion of HCAEC is different



**Figure 1** Invasion of human coronary artery endothelial cells by black pigmenting bacteria associated with endodontic infections. *P. gingivalis* 381 and *E. coli* MC1061 were used as the positive and negative controls, respectively, since their invasive ability in these cell lines have been characterized previously (Dorn *et al.* 1999). Error bars represent the standard deviation ( $n = 3$ ).



**Figure 2** Transmission electron micrograph of internalized *P. endodontalis*. Internalized *P. endodontalis* (arrow) in HCAEC following a 90-minute infection.

from the *P. gingivalis* vacuole. *P. gingivalis* is localized within autophagosomes, whereas *P. endodontalis* is located in large endosomes contained by a thin membrane. *P. endodontalis* ATCC 35406 also invaded CASC (1.1 ± 0.04 × 10<sup>4</sup>) in the antibiotic protection assay.

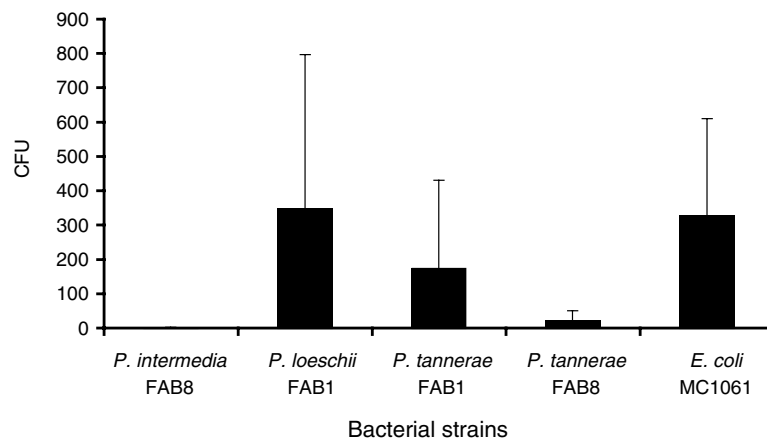
All of the other strains tested had invasion levels below the non-invasive control *E. coli* MC1061. *P. endodontalis* strain R-41 and strain H11a–e were not invasive. Thus, the invasion ability of *P. endodontalis* varies between the strains. The type strain of *P. nigrescens*, the most frequently isolated BPB from endodontic lesions and a close relative of *P. intermedia*, was also not invasive.

Microorganisms from infected root canals were isolated, identified, and tested for their invasive ability. Four strains were positively identified as *P. intermedia*, *Prevotella loeschii*, and *Prevotella tannerae* (from two different patients). None of these four strains were invasive (Fig. 3). A fifth strain was isolated and was also non-invasive; however, tests could not definitively determine whether it was *Prevotella melaninogenica* or *P. nigrescens*. None of the bacteria isolated from infected root canals were invasive in the antibiotic protection assay.

## Discussion

Microorganisms from oral focal infections may disseminate throughout the body and subsequently colonize extraoral tissues. During septicaemia, microorganisms from the oral cavity may attach to and invade sites along the arterial tree. This theory was originally formulated to address the putative association between periodontal disease and cardiovascular disease. The theory could also apply to microorganisms associated with endodontic infections due to bacteraemia after endodontic therapy (Debelian *et al.* 1995, Debelian *et al.* 1998). The interactions between the bacteria and the vascular cells could initiate, or more likely, exacerbate the inflammatory lesion of atherosclerosis (Ross 1999).

In contrast to the focal infection theory, microorganisms from endodontic infections may not be seeding distant sites in the body, but rather the dental pulp may be infected by a general bacteraemia (Drancourt *et al.* 1998, Aboudharam *et al.* 2000). Therefore, bacteria found in the dental pulp could be a reflection of microorganisms found systemically. These bacteria may be indicators of pathogens causing morbidity at extraoral sites. More specifically, invasive bacteria in the dental



**Figure 3** Invasion of human coronary artery endothelial cells by clinical isolates. Bacteria were isolated from infected root canals and tested using the antibiotic protection assay. Error bars represent the standard deviation ( $n = 3$ ).

pulp may be indicative of bacteria causing disease at the endothelium.

The invasion ability of strains of *P. endodontalis* in this study was heterogeneous. Of the three strains tested, only *P. endodontalis* ATCC 35406 was invasive. This finding was not unexpected, since previous studies have demonstrated that the invasion abilities of both *P. gingivalis* and *P. intermedia* vary amongst the different strains (Dorn et al. 1998). Conversely, certain strains of *P. loeschii*, *P. nigrescens*, and *P. tannerae* that were not tested in this study may be invasive. The different strains of BPBs from the oral cavity vary in their ability to invade non-phagocytic cells. The intracellular location of *P. endodontalis* was different from that of *P. gingivalis*. Although both are invasive, the two microorganisms have evolved different mechanisms of controlling their intracellular environment in order to survive (Hackstadt 2000).

In summary, we have demonstrated that some, but not all, microorganisms associated with endodontic infections can invade human coronary artery endothelial cells (HCAEC) and coronary artery smooth muscle cells (CASM). A next step would involve animal models to determine whether the bacteraemic conditions would produce the same effect *in vivo*. If the hypothesis regarding atherosclerotic exacerbations caused by the direct interactions between oral bacteria and vascular cells is correct, then microorganisms in the pulp chamber may represent a source of infectious organisms that contribute to the pathogenesis of CHD.

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